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Supported Ionic Liquid-Like Phases (SILLPs) for enzymatic processes: Continuous KR and DKR in SILLP–scCO$_2$ systems†

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The immobilisation of *Candida antarctica* lipase B (CALB) onto SILLPs has been studied in order to obtain the maximum activity, selectivity and stability of the resulting supported biocatalyst for the KR of *rac*-1-phenylethanol by flow processes using scCO$_2$. This involves the use of hydrophilic SILLPs (chloride anion) with high loadings of IL moieties and CALB. The combination of the immobilized biocatalyst with an acidic zeolite has allowed us to carry out DKR processes. Excellent results in terms of yield (92%) and enantioselectivity (>99.9% ee) are obtained by the use of a "one-pot" reactor containing a mixture of both catalyst particles under scCO$_2$ flow conditions (50 °C, 10 MPa), resulting in an environmentally friendly process with high productivity and enantioselectivity.

Introduction

Ionic liquids (ILs) are simply salts with melting points below 100 °C, usually close to room temperature.[1] Their interest as green neoteric solvents resides in their negligible vapour pressure, their excellent thermal stability, and their high ability to dissolve a wide range of organic and inorganic compounds, including gases and their non-flammable nature.[2] Thus, ILs can be used to mitigate the problem of volatile organic solvent emission to the atmosphere and have been implemented as environmentally friendly alternative solvents for various processes.[3] Thus, ILs have emerged as exceptionally interesting non-aqueous reaction media for enzymatic transformations,[4] mainly for asymmetric synthetic transformations, due to the growing demand for enantiopure pharmaceuticals.[5]

In the search for cleaner biocatalytic processes, the use of supercritical fluids (SCFs) is another interesting alternative.[6] However, in some cases, the poor stability exhibited by enzymes in SCFs, particularly in scCO$_2$, is the main drawback for the application of biocatalytic processes in SCFs.[7]

Alternatively, different approaches based on the coating of biocatalysts by ILs or on the suspension of the enzyme in the IL have been developed to improve the stability and activate the enzyme.[8,9,10] Thus, the synergistic combination of both SCFs and ILs for enzyme catalysis can led to greener and highly efficient processes. Indeed, the coating or suspension of biocatalysts in an IL has proved to be the best approach to protect enzymes against these adverse effects of scCO$_2$.[10] The high solubility of the scCO$_2$ phase in ILs and, simultaneously, the low solubility of the ILs in the in the scCO$_2$ phase, allows the supercritical fluid to be used as the medium to deliver the products to the enzyme immobilised in the IL and to extract the obtained products from them. Therefore, it is easy to combine reaction and separation protocols, leading to integral greener bioprocesses in non-aqueous media.[11]

The aim of this work is to push towards the efforts to design greener enzymatic processes in IL/scCO$_2$ biphasic systems. The catalytic system is based on an enzyme (*Candida antarctica* Lipase B, CALB) immobilised by non-covalent attachment onto different polymeric Supported Ionic Liquid-Like Phases (SILLPs). SILLPs are polymeric matrices modified with IL-like units.[12] They can be regarded as "solid ionic solvents" or as nanostructured materials with microenvironments of tunable polarity similar to those provided by bulk ionic liquids.[13] The solid nature of the SILLPs facilitates the separation processes, allowing the development of continuous flow processes and avoiding accidental spills. This is important in the light of the toxicological and ecotoxicological concerns recently raised for some ILs.[14] In addition, it reduces the cost of the process, as smaller amount of ILs need to be employed.[15]

We have shown that the adsorption of a lipase onto this linked IL-like phase provides excellent immobilized biocatalysts. Indeed, these show enhanced activity and increased operational stability for the continuous synthesis of citronellyl butyrate in scCO$_2$, compared with the original strategy based on enzymes coated with ILs.[16] The present study reports on the use of different CALB–SILLPs as biocatalysts for a stereospecific reaction model such as the kinetic resolution (KR) of *rac*-1-phenylethanol in both batch and continuous process. The biocatalysts show good performance in both a conventional organic solvent (hexanes) and in supercritical carbon dioxide (scCO$_2$). In addition, the continuous one-pot dynamic kinetic resolution (DKR) of this substrate in scCO$_2$ by *in situ* racemization of the

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undesired enantiomer is also reported with good chemical yield (>85%) and excellent enatiopurity (>99% ee).

Experimental

Safety note

Experiments with $scCO_2$ involve high pressure and should only be carried out in equipment with the appropriate pressure rating.

Materials

The aqueous solution of *Candida antarctica* lipase B (Novozym 525 L, EC 3.1.1.3) was a gift from Novozymes S.A. Acid zeolites CP811C (zeolite β, 620 m^2/g), CBV720 (zelolite Y, 720 m^2/g) and CP811E (zeolite β, 600 m^2/g) were obtained from Zeolysts International (Valley Forge, PA, USA). Substrates, solvents and other chemicals were purchased from Sigma-Aldrich-Fluka Co. (Madrid, Spain), and were of the highest purity available.

Preparation of Supported Ionic Liquid-Like Phases 4–6 (SILLPs)

The protocol for preparation of the supported ionic liquid phases **4–6**, bearing different covalently attached IL-like moieties, has been previously reported in detail.[12] All these materials were characterized by various techniques, including FT-IR, Raman microspectroscopy, and elemental analysis, which confirmed their expected structures. The quantitative exchange of the chloride anion in the metathesis reaction was confirmed by XPS analysis by the absence in the exchanged SILLPs of the band corresponding to the chloride group.

CALB immobilization onto SILLPs

The immobilized enzyme derivatives were prepared by simple adsorption of 1 mL of an aqueous solution of CALB (18.2 mg/mL) onto SILLPs **4–6** (500 mg). The mixture was shaken for 6 h at room temperature to adsorb the enzyme. The supernatant was recovered and the support washed with water to remove non-adsorbed enzyme. The polymer was frozen at –60 °C and lyophilised. The supernatant and washing fractions were collected and used to quantify the amount of immobilized protein by Lowry's modified method.[17] All the supported enzymes were stored under controlled water activity (Aw) conditions over LiCl (Aw = 0.11) in desiccators for 48 h at room temperature prior to use.

Batch kinetic resolution of *rac*-1-phenylethanol in hexane

Reactions were carried out in 1 mL screw-capped vials with Teflon-lined septa containing 600 μL of substrate solution (450 mM *rac*-1-phenylethanol, 950 mM vinylpropionate) in dry hexanes. The reaction was started by adding the CALB–SILLP (**7–11**) (25 mg), and run with magnetic stirring at 50 °C (oil-bath) for 8 h. At regular time intervals, 20 μL aliquots were taken, suspended in 480 μL hexanes containing 20 mM butyl butyrate (internal standard), and then analysed by GC.

Profiles of (*R*)-1-phenylethylpropionate concentration with respect to time were used to quantify the reaction rates of the system. Conversion was calculated as $\%c = ee_S/(ee_S + ee_P) \times 100$, where the subscripts S and P stand for substrate and product, respectively.[18] Enantiomeric excesses for the synthetic products were calculated as:

$ee_S = [(\%(S)\text{-}\mathbf{12} - \%(R)\text{-}\mathbf{12})]/[\%(S)\text{-}\mathbf{12} + \%(R)\text{-}\mathbf{12})] \times 100$

$ee_P = [(\%(S)\text{-}\mathbf{15} - \%(R)\text{-}\mathbf{15}]/[(\%(S)\text{-}\mathbf{15} - \%(R)\text{-}\mathbf{15})] \times 100$

One unit of synthetic activity was defined as the amount of enzyme that produces 1 μmol of (*R*)-1-phenylethylpropionate per min. All the experiments were carried out in duplicate

Kinetic resolution of *rac*-phenylethanol in $scCO_2$

The immobilized enzymes (300 mg) were packed into a stainless-steel column ($\frac{1}{4}$ inch external diameter, 10 cm length) and then introduced into an oven at 50 °C. The system was equipped with a supercritical pump (JASCO, model PU-1580-CO_2), needle valves, filter and a back-pressure regulator (JASCO, model BP 2080). The reactor was run by the continuous pumping of $scCO_2$ at 10 MPa through the packed immobilized enzyme, then the back-pressure regulator, and finally bubbling through a capillary tube at 1 mL/min total flow in a controlled amount of n-hexane placed on ice-bath. The KR process was carried out by the continuous pumping of an equimolar solution of *rac*-1-phenylethanol and vinyl propionate at 21.2 μmol/min mass flow-rate, by using a HPLC pump (model LC-10AT, Shimadzu Europe, Duisburg, Germany) for 4 h. Collected samples were analyzed by GC, the substrates/products mass-balance from the outlet being consistent with the substrate mass-flow inlet.

Dynamic kinetic resolution of *rac*-phenylethanol in $scCO_2$

Two fractions (150 mg) of immobilized derivative, and one fraction of acid zeolite (100 mg of CP811C, CBV-720 or CP811E coated with 150 μL of [Btma][NTf_2]) were packed in three separated columns. The three columns connected in series (immobilized lipase–acid catalyst–immobilized lipase) were operated in a continuous $scCO_2$ flow for 4 h cycles by using the system and procedure described above.

GC analysis

Analysis was performed with a Shimadzu GC-2010 (Shimadzu Europe, Duisburg, Germany) equipped with an FID detector. Samples were analyzed on a Beta DEX-120 column (30 m × 0.25 mm × 0.25 μm, Supelco), using He as the carrier gas and a FID detector, as described previously[18] Retention times of compounds are as follows: vinyl propionate (3.1 min), propionic acid (5.6 min), butyl butyrate (internal standard, 7.1 min), (*R*)-1-phenhylethanol (14.8 min), *S*-1-phenhylethanol (15.5 min), (*S*)-1-phenylethyl propionate (18.2 min) and (*R*)-1-phenylethyl propionate (18.6 min).

Results and discussion

Synthesis and characterisation of CALB–SILLPs

The polymers modified with IL-like moieties were synthesized by grafting of butyl imidazole onto a commercial bead-type Merrifield resin (**1–3**) leading to the corresponding SILLPs **4–6** (Scheme 1).[12] Alternatively, Merrifield-type polymers with different loadings (1.1 and 4.3 meq Cl/g) crosslinking degrees and morphologies (gel: 2% DVB or macroporous: 5.5% DVB)

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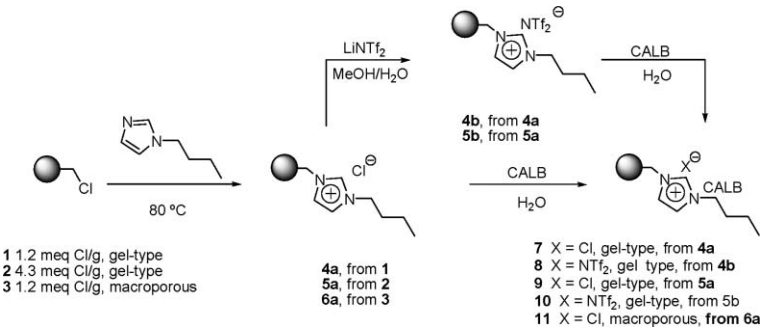
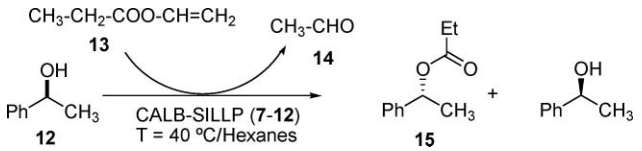


Scheme 1 Synthesis of the CALB–SILLPs.

were employed. Details about the synthesis and characterization of these compounds have been published by us previously.[12,16] The metathesis of chloride by NTf_2^- afforded more hydrophobic SILLPs (**4a–5a**).

The immobilisation of the enzyme (CALB) was carried out by adsorption from an aqueous solution of the enzyme as described in the Experimental section. Table 1 summarises the immobilisation yield reached for the different supports assayed. As can be seen, all the supports were able to adsorb some protein, with the percentage of immobilisation ranging from *ca.* 7 to 60%. This difference in the immobilisation yield clearly depends on both the nature of the support and the amount and type of the IL-like moieties present in the SILLPs. Thus, hydrophilic SILLPs having chloride as counterion (**4a** and **5a**) adsorbed less protein than the hydrophobic ones (Table 1, Entry 1 and 3 *vs.* 2 and 4). In contrast, the macroporous resin (**6a**) adsorbed a significantly higher percentage of enzyme than the gel-type analogue (Table 1, Entry 1 and 3 *vs.* 5). In the case of the hydrophobic SILLP (**4b** and **5b**) the adsorption depends of the loading of the ionic liquid units. Thus, an increase in the loading of the ILs from *ca.* 21% to *ca.* 78% by weight, led to a two-fold increase in the enzyme loading (from 11 to 21 mg of protein/g of SILLP). However, a similar trend was not observed when the SILLPs with Cl as the counterion were used. In this case, the high content in hydrophobic aminoacids placed at the surface of CALB could be related to these results: the more hydrophobic microenvironment for the SILLP, the better the immobilization yield.[19]

The ability of CALB–SILLPs **7–11** to catalyse the KR of *rac*-1-phenyletanol with vinyl propionate (Scheme 2) was examined



Scheme 2 Kinetic resolution of *rac*-1-phenylethanol.

first. In all the cases the reaction was carried out in hexanes at 50 °C using the same weight of polymer independent of protein loading, and 24 h of reaction time. All the supported enzymes were able to catalyse the selective formation of (*R*)-1-phenylethyl propionate with excellent enantiomeric excess (99.9% ee). However, the catalytic efficiency of the immobilised enzymes was clearly dependent on the characteristics of the SILLPs. In general, the catalysts CALB–SILLP **7** and **8** with low IL-like loadings led to significantly lower yields (18% and 13% yield, respectively) than the analogues with higher IL loading (50% for **9** and 36% for **10**). The protein adsorbed in macroporous SILLP (**11**) also showed an excellent activity and enantioselectivity. Regarding the activity (U per g support), it was found that the best immobilised catalyst, for the conditions assayed, was CALB-SILLP **10** due to its higher protein loading compared with the other CALB–SILLPs. However, if the specific activity (U/mg of enzyme) was considered, the picture was quite different. In this case, the more active catalyst was CALB–SILLP **9**.

In general, some trends can be detected: i) enzymes supported on hydrophilic supports show higher specific activity than those on hydrophobic supports,[20] ii) higher IL-like loadings led to significant improvement of the specific activity. Both tendencies

Table 1 Immobilisation and catalytic parameters for *Candida antarctica* lipase B adsorbed onto different supports[a]

Entry	R	X⁻	Loading (meq IL per g)[b]	CALB–SILLP[c]	% IL	Immobilised protein[d]	Immobilisation yield (%)[e]	*c* (%)	ee_P[f] (%)	*E*	Activity[g]	Specific activity[h]
1	C_4H_9	Cl^-	0.76	**7** (g)	21.0	2.6	7	18	99.9	244	18	7.0
2	C_4H_9	NTf_2^-	0.60	**8** (g)	31.3	11.4	32	13	99.9	230	15	1.3
3	C_4H_9	Cl^-	2.33	**9** (g)	64.5	3.0	8	50	99.9	1510	68	22.5
4	C_4H_9	NTf_2^-	1.51	**10** (g)	78.7	21.2	59	36	99.9	346	307	14.4
5	C_4H_9	Cl^-	0.85	**11** (m)	23.5	17.5	48	50	99.9	1510	195	11.2
6	—	—	—	—	—	15.0	41	—	—	—	0.02	0.3

[a] 100 mg of the support were suspended in 200 μL of a solution of CALB (18.2 mg/mL). Supplied protein: 36.3 mg per g support. [b] Based on elemental analysis. [c] Type of resin: (g) = gel, (m) = macroporous. [d] mg of protein per g support. [e] % immobilisation yield = (mg immobilised protein per g support)/(mg supplied protein per g support) × 100, calculated by Lowry's method. [f] ee_P = [(%(*S*)-**15** – %(*R*)-**15**]/[(%(*S*)-**15** – %(*R*)-**15**)] × 100. [g] Activity = U (μmol product per mL per min) per g support. [h] U per mg protein.

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highlight the importance of the enzyme–IL interactions, as well as the preservation of the water-shell around proteins, to provide active and stable biocatalysts suitable for being used in $scCO_2$. Thus, the SILLPs having NTf_2 as the counterion showed an eleven-fold enhancement in the specific activity for a two-fold protein loading increase. It is noteworthy that, for the gel-type hydrophilic SILLPs, a three-fold increase of the specific activity was observed for almost the same protein loading (*ca.* of 3 mg per g support); iii) for catalysts **7** and **11** with similar IL loadings and different polymeric morphology, the macroporous resin yielded a slightly higher specific activity; iv) the specific activity of the hydrophobic resins (**8** and **9**), when compared with their protein content, seems to indicate an inactivation of the enzyme by aggregation or by perturbation of the secondary protein structure for higher loadings, v) although resin **3** was able to immobilise *ca.* 40% of the protein supplied, the specific activity was very small. The last two observations illustrate the importance of having a large IL/enzyme ratio to stabilise the immobilised enzyme.

Indeed, the specific activity of our supported enzyme CALB–SILLP **11** with respect to a commercial one (Novozyme 435 without coating),[21] calculated as U per mg of supported enzyme, is higher than that found for the commercial catalyst under the same experimental conditions and using the same amount of supported biocatalyst (*ca.* 11 *vs.* 5 U per mg supported enzyme, respectively).[22]

Most likely, a rational explanation for the different results observed for the CALB–SILLPs can be related to the variation of the secondary structures of the protein produced by the immobilisation onto the different supported ionic liquids. The infrared spectra of the proteins, in particular the amide I region at 1700–1600 cm^{-1}, can provide some valuable information about the protein's secondary structure in terms of α-helix, β-sheets, β-turns and non-ordered or irregular structures.[23] Therefore, the FT-ATR-IR spectra of CALB–SILLP **9**, CALB–SILLP **10** and Novozyme 435 were recorded and analysed by deconvolution of the amide I region (see ESI†).

Both FT-ATR-IR spectra of CALB-SILL-**10** and Novozyme 435 showed a single peak at 1658 cm^{-1}. In addition, bands at *ca.* 1670 cm^{-1} (associated with β-turns not associated with hydrogen bonds), and at 1690 cm^{-1} (which could be related to the formation of aggregates), clearly indicate the structural changes of the lipase. On the other hand, the spectrum of CALB–SILLP **9** showed a band at 1649 cm^{-1}, which can be associated with the α-helix structure, while peaks at 1660 and 1667 cm^{-1} are characteristic of β-turns.[24] This indicates that in the two first cases the immobilisation is be accompanied by a loss of both α-helix and β-turn substructures. These structural changes may well explain the low specific activity of these two supported biocatalysts compared with CALB–SILLP **9**.[25] In contrast, it seems that the protein in the SILLP bearing chloride as the counterion (CALB–SILLP **9**) preserves more efficiently the active protein structure. The combination of hydrophilic IL-like moieties and high IL/enzyme ratios clearly contribute to the stabilization of the structure of the enzyme, as has been observed in bulk ILs.

A study by differential scanning calorimetry (DSC) of the CALB immobilised onto SILLPs (**5a–b**) and the commercial immobilized CALB (Novozyme 434) was also carried out to characterise the effect of the supported ILs on the thermal denaturation of the protein. The DSC traces showed a significant shift of the maximum temperature observed from 52 °C (Novozyme 435) to 57 °C (CALB–SILLP **10**, 4.3 meq Cl/g, R = C_4H_9, X = NTf_2) and 68 °C (CALB–SILLP **9**, 4.3 meq Cl/g, R = C_4H_9, X = Cl). Thus, the nature of the polymer and the anion contribute to the stabilization of the supported protein. The results from both FT-ATR-IR and DSC proved a significant effect of the SILLPS over the stabilization of the secondary structure of the protein, the support having chloride as the counterion and high IL-like loading being the most efficient in terms of specific activity and thermal stability.

Once we had proven that the SILLPs can be used as "solid ionic solvents" for the immobilisation and stabilisation of the CALB leading to efficient biocatalysis for the KR of *rac*-1-phenylethanol, we focused on developing the system further. In this regard, we studied several variations of the immobilisation protocol to achieve the highest yield of immobilisation for each resin, trying to optimise the activity (U per g support). For this purpose, we decided to study the highly cross-linked (macroporous) polymer SILLP **6**, which has a higher mechanical strength than gel-type resins, and accordingly might be more suitable for flow processes.

Table 2 summarises the efficiency of the immobilization process onto SILLP **6** as a function of the supplied amount of protein (from 5.5 to 63 mg of protein per g of support). As can be seen, by increasing the supplied protein, the enzyme loading in the resin increased, which led to a corresponding enhancement of the biocatalytic activity (from 24 to 271 U per g support) for the KR of *rac*-**12**. The same excellent stereoselectivity was achieved for all the cases (>99.9% ee). It is noteworthy that the specific activity remained practically constant. Only a slightly lower enzymatic efficiency was observed for the highest

Table 2 Optimisation of the immobilisation of CALB onto SILLP **6** (CALB–SILLP **11**)[a]

Entry	Protein supplied[b]	Immobilised protein[c]	Activity (U per g support)[c]	Specific activity (U per mg protein)
1	5.5	0.9	24	25.1
2	10.8	1.4	35	24.8
3	25.4	5.9	122	20.7
4	38.0	10.0	220	22.0
5	63.4	14.0	271	19.3

[a] The KR was carried out in n-hexane at 40 °C using a *rac*-1-phenylethanol–vinyl propionate ratio of 1 : 2. [b] 100 mg of the support was suspended in 200 μL of an aqueous solution with variable concentrations of CALB supplied (mg per g support). [c] mg CALB per g support calculated by Lowry's method: % immobilisation = (mg immobilised protein per g support)/(mg supplied protein per g support) × 100. [d] U = μmol product per mL per min.

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protein loading. Furthermore, CALB–SILLP **11** with a loading of *ca.* 10 mg of protein per g support could be reused for three consecutive batch cycles without any significant loss of activity or stereoselectivity. When comparing the data gathered in Tables 1 and 2, it can be seen that the supported biocatalyst CALB–SILLP **11** prepared by the optimised protocol provides comparable activities and similar specific activities to the best gel-type supported enzyme.

In order to develop a more sustainable and efficient system, we decided to test the ability of CALB–SILLPs to catalyse the KR of *rac*-**12** on a continuous flow system. The solid nature of the CALB–SILLP **11** (10 mg of protein per g support) allows one to easily design a fixed-bed reactor to perform KR under flow conditions.[26] In addition, this set-up facilitates the substitution of a traditional, relatively toxic, solvent (n-hexane) by a non-toxic one such as $scCO_2$. Fig. 1 depicts the profiles of productivity (μmol per g support per min) of (*R*)-1-phenylethyl propionate *vs.* time reached for a continuous KR of *rac*-**12** in $scCO_2$ at 50 °C and 10 MPa. The productivity can be easily improved by adjusting the mass flow rate of the organic stream from 3 to 15 μmol min^{-1}, as a consequence of the high enzyme loading of the support. The yield of ester product was *ca.* 50% after reaching the steady state, for all the conditions assayed. The synthetic product **15** was obtained with an excellent enantiopurity (>99.9% ee), while (*S*)-ester product **15** was never detected. The system maintained the efficiency even for an extended period of time (up to 6 days of continuous use).

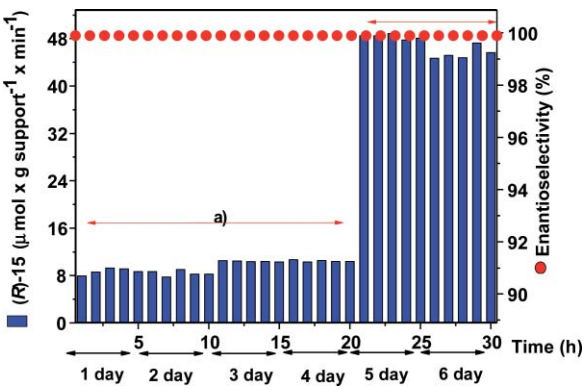

Fig. 1 Profiles of productivity and ee of (*R*)-1-phenylethyl propionate *vs.* time on stream for the continuous KR of *rac*-**12** in $scCO_2$ at 50 °C and 10 MPa: a) 3 μmol min^{-1}; b) 15 μmol min^{-1}.

We have developed a stable, efficient and stereospecific continuous KR of *rac*-**12** immobilising the CALB onto SILLPs and using $scCO_2$ as vector to deliver the substrates to the active sites and to extract the products. The nature of the support may affect the partitioning of water and substrate molecules with the bulk medium, and so, simultaneously, to favour mass-transfer phenomena and to preserve the hydration shell of the enzyme. Thus, for instance, the continuous flow of $scCO_2$ cannot extract the free water molecules from the CALB–SILLP, reducing in this way the potential hydrolytic reaction. Indeed, very low levels of propionic acid (<1%) were observed in all the cases.

The modified polymer with IL-like groups provides the required microenvironment for the stabilisation of the enzyme leading to active, stable and enantioselective biocatalysts. Furthermore, the use of CALB–SILLPs reduces significantly the amount of ionic liquid used as compared with either bulk homogeneous ionic liquid[27] or enzymes coated with IL layers.[10] Thus, for instance, for coated enzyme supported on different materials, a 1 : 1 ILs–support ratio is required to avoid deactivation.[11d] The approach here reported does not require an additional IL layer to stabilise the enzyme against deactivation, being stable for a long period (Fig. 1). This leads to a significant minimisation of cost and hazards related to the use of bulk ILs. Furthermore, coated supported enzymes with IL layers may leach by either physico-mechanical removal by convention or by partial dissolution of the ILs in the liquid reaction phase. These phenomena are not possible in CALB–SILLPs, as the IL-like phases are covalently linked to the support.

A maximum theoretical yield of 50% can be obtained for the KR procedure. However, DKR processes, in which *in situ* racemisation of the unwanted enantiomer is coupled with KR, is an attractive method to overcome this limitation.[28] Although some improvements have been made in this direction, the DKR of aliphatic alcohols using a catalyst under environmentally friendly and mild conditions remains a challenge.[29] The key issue is related to the use of simple experimental conditions compatible for both the biocatalyst and the chemocatalyst. The use of either an immobilized transition metal catalyst or a heterogeneous acid catalyst for the alcohol racemization simplified the processes significantly and minimized the possible (mutual) deactivation during the DKR process.[30] Acid catalysts (Zeolites, Amberlite, Nafion, *etc.*) are by far a simpler approach, being cheap and readily available catalysts that have been used for the DKR of phenyl ethanol. The main problem in combining the acid-catalyzed racemisation and the enzymatic resolution of **12** by esterification is that the reactions have to occur under aqueous and water-free conditions respectively. Hence, to get good enantioselectivity in DKR systems using acid catalysts (mainly zeolites), the zeolites and the biocatalysts need to be physically isolated.[31]

Taking into the account these considerations, we assayed the DKR using the configuration shown in Fig. 2. Two fixed-bed reactors loaded with the immobilized enzyme (biocatalytic reactor, CALB–SILLP **11**, 150 mg) and an additional one with an acid zeolite (chemical racemisation catalyst, 100 mg) were assembled with the configuration biocatalyst–chemocatalyst–biocatalyst. It was decided to use separate columns for each catalyst to isolate them, avoiding any possible enzyme deactivation, which was observed in previous experiments where the enzymatic catalyst and the acid catalyst were mixed together.[32] In addition, this particular reactor configuration easily allows us to replace the acid catalyst reactor, facilitating the screening of different zeolites (CP811C, CBV720 and CP811E), which were initially selected as chemocatalysts as they have proved to be good catalysts for the racemisation of enantiopure phenyl ethanol.[33]

It should be also noted that, for this particular approach, the (*R*)-**15** yield may only reach 100% by coupling in series several enzymatic and acidic catalytic columns, following a dichotomic progression. Thus, we must bear in mind that only a maximum yield of 75% can be reached with our actual experimental configuration. Fig. 3 depicts the results obtained for the continuous DKR at 50 °C and 10 MPa.

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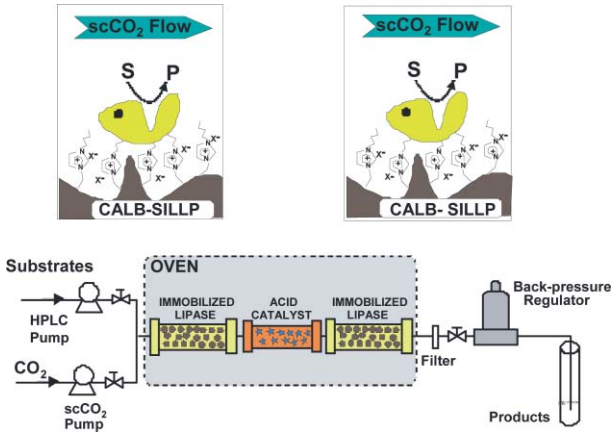

Fig. 2 Experimental set-up for the continuous DKR of *rac*-**12** using a combination of consecutive biocatalytic–chemocatalytic–biocatalytic reactors.

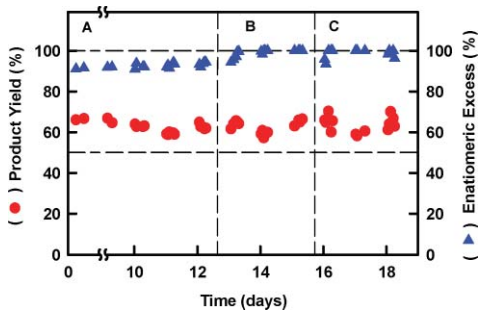

Fig. 3 Profiles of (*R*)-1-phenylethyl propionate yield (●) and enantiomeric excess of ester products (▲) during the continuous DKR of *rac*-1-phenyl ethanol catalyzed by CALB–SILLP **11** and zeolites CP811C (A), CBV720 (B) and CP811E (C) as acid catalysts in $scCO_2$ at 50 °C and 10 MPa.

The DKR was successful in all cases. The total yield of the *R*-ester product was *ca.* 63% for all the time-course profiles, which corresponded with *ca.* 84% of the possible *R*-ester product. The biocatalytic reactors loaded with the same sample of CALB–SILLP were used for all acid catalysts screening experiments. In addition, the CALB–SILLP maintained its activity in continuous operation for 19 days. This excellent operational stability was in agreement with previous results of enzymes in ionic liquids environments and with enzymes immobilized onto SILLPs.[16]

The use of different zeolites led to changes in the enantioselectivity of the process, mainly due to the differing ability of zeolites to catalyze the non-stereoselective transesterification reaction that produces the *S*-ester. In this way, the use of the CP811C zeolite reduced the ee of the *R*-ester product to 92%, while the best results were obtained for both CBV720 and CP811E zeolites, that reached the maximum ee (>99.9%). Furthermore, the presence of propionic acid was observed in the $scCO_2$ flow when an acid catalyst column was included into the reactor configuration. In spite of the dryness of the $scCO_2$, the residual water content in the porous structure of the chemocatalyst could be involved in this undesired activity. This shows the necessity for strict control of the water content of these materials before starting the DKR processes.

Once we had proven that the DKR based in the combination of CALB–SILLPs and zeolite can be achieved with excellent enantioselectivities, we tried to develop our system further using a single reactor, in which both bio- and chemocatalyst are mixed together to perform a "one-pot" DKR in $scCO_2$ (see Fig. 4).

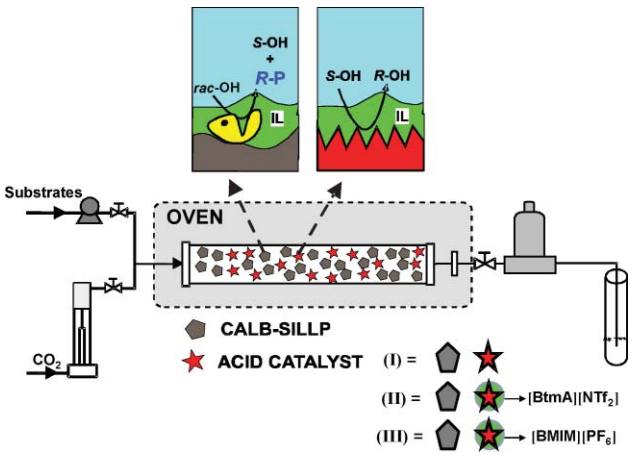

Fig. 4 Experimental set-up for the continuous "one-pot" chemoenzymatic DKR of *rac*-**12**.

The "one-pot" DKR using a combination of CALB–SILLP **11** and zeolite (CP811E-150) in the same reactor yielded an unselective process (63% ee), both enantiomers of the ester **15** being observed (see Entry 1, Table 3). The low selectivity can be attributed to direct esterification of *rac*-**12** catalysed by the zeolite. It has been proved that the IL-coating of H-β zeolites with a hydrophobic IL significantly reduces its racemisation activity and the side reaction (acylation).[34] This is due to the fact that the IL monolayer increases the mass-transfer of the reactants to the active sites and also isolates the acid sites from the eventual presence of water in the system. Therefore, several combinations of CP811E-150 coated with two different ILs ([Btma][NTf_2] and [BMIM][PF_6]) and CALB–SILLP **11** (see Table 3) were tested in the DKR. In both cases, good initial yields of *ca.* 70% were observed. Additionally, the process took place with a significant increase of the enantioselectivity. Indeed, only the *R*-ester **15** was detected (>99.9% ee). Finally, the flow rate of the system was reduced in order to increase the yield of the

Table 3 Continuous "one-pot" DKR of *rac*-**12** in $scCO_2$[a]

		Composition (outlet) (%)				
Entry	Set-up[a]	(*R*)-**12**	(*S*)-**12**	(*R*)-**15**	(*S*)-**15**	ee (%) of **15**
1	I	7	11	48	11	63
2	II	8	18	74	0	99.9
3	III	6	26	68	0	99.9
2	II[b]	2	6	92	0	99.9

[a] I: 600 mg of CALB–SILLP **11** and 400 mg of CP811E-150. II: 600 mg of CALB–SILLP **11** and 200 mg of CP811E-150 + 200 μl [BtmA][NTf_2]. III: 600 mg of CALB–SILLP **11** and 200 mg of CP811E-150 + 200 μl [BMIM][PF_6]. **12** = 1-phenylethanol; **15** = phenylethyl propionate; [BMIM] = butylmethylimidazolium; [BtmA] = butyltrimethylamonium. [b] Half the mass flow rate (10.6 μmol min^{-1}) was used.

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process. Thus, a yield of the *R*-ester of up to 92% could be reached with an excellent enantioselectivity >99.9%

Conclusions

The results here presented clearly highlight the great potential of supported IL-like phases (SILLPs) as "solid solvents" for the direct immobilization and stabilization of enzymes and for the development of new biocatalytic systems. The efficiency of the resulting biocatalysts, in terms of both activity and specific activity, for the KR of *rac*-1-phenylethanol using CALB is affected by several factors, as are the nature and morphology of the polymeric material, the loading of IL-like moieties, the loading of the enzyme and the chemical structure of the IL-like fragments, in particular the nature of the anion (which is essential to define the hydrophilic/hydrophobic balance of the corresponding material). All those parameters can be easily adjusted and optimised. Best results are obtained for hydrophilic IL-like fragments containing chloride as the anion. In the same way, high loadings of both IL-like moieties and enzymatic units are preferred, although keeping a relatively high IL-like/enzyme ratio favour maintaining the active native secondary structure of the enzyme. This is revealed by the long-term stability of the resulting supported biocatalyst and in the appreciable increase in the protein denaturation temperature.

The use of a macroporous resin facilitates the preparation of biocatalytic minireactors for continuous-flow applications in $scCO_2$ that can be used for more than one week without any appreciable reduction in their efficiency. Excellent yields (50%, the maximum attainable for a KR) and enantioselectivities (>99.9% ee) are continuously obtained at 50 °C and 10 MPa. The combination of this biocatalytic system with an acid catalyst has allowed us to develop different DKR processes. The assembly of three columnar reactors (biocatalyst–chemocatalyst–biocatalyst) produces a system that can work efficiently for more than 3 weeks without a reduction in performance, while raising the yields to more than 60% and allowing the identification of the most efficient acid catalyst able to maintain an excellent enantioselectivity. A "one-pot" single columnar minireactor can be assembled by loading the corresponding column with a mixture of CALB–SILLP **11** and CP11E-150. To achieve a selective process, the zeolite needs to be coated with a small amount of an IL. Optimisation of this approach has allowed us to increase the yields of the desired product up to 92% with enantioselectivities higher than 99% ee.

The system here reported represents an important improvement compared with previously reported DKRs, either in conventional or neoteric solvents (see the ESI† for a comparison of different supported catalysts used for DKR). DKR, when performed in a conventional solvent using a homogeneous organometallic catalyst for racemisation, usually requires anhydrous conditions, inert atmospheres, the addition of a base, and batch operation for extended periods of time. Those limitations are overcome in the present system, which is an evolution of previous systems that, in a simple way, allows us to obtain a high productivity in short periods of time and with the concomitant savings not only in terms of the economical viability, but also environmental factors.[10]

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